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<b>(21) International Application Number:</b> PCT/US95/12381 <b>(22) International Filing Date:</b> 29 September 1995 (29.09.95)  <b>(30) Priority Data:</b> 08/315,336                      30 September 1994 (30.09.94)      US 08/334,606                      4 November 1994 (04.11.94)        US  <b>(71) Applicant:</b> RUTGERS, THE STATE UNIVERSITY [US/US]; Old Queens, Somerset Street, New Brunswick, NJ 08903 (US).  <b>(72) Inventors:</b> TRIMMER, William, Stuart; 58 Riverview Terrace, Belle Meade, NJ 08502-3218 (US). LING, Peter, Ping; 4 Ruthies Run, Cranbury, NJ 08512 (US). HASHMI, Sarwar, Apartment 485, 117 Royal Drive, Piscataway, NJ 08854 (US). GAUGLER, Randy; 25 Quince Place, North Brunswick, NJ 08902 (US).  <b>(74) Agents:</b> FOLEY, Shawn, P. et al.; Lerner, David, Littenberg, Krumholz & Mentlik, 600 South Avenue West, Westfield, NJ 07090 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DIRECT INTRODUCTION OF FOREIGN MATERIALS INTO CELLS  <b>(57) Abstract</b>  <p>Disclosed is a simple, economical, and precise method of introducing a biological material into a predetermined target cell population. The method comprises the steps of providing (a) a plurality of biologically inert microprobes positioned on a support, (b) a solid or quasi-solid mass of the target cells defining an interface with the microprobes, and (c) a biological material at the interface, and then physically contacting the cells with the microprobes to cause the microprobes to non-lethally pierce the cell walls and/or membranes of the cells. The microprobes are preferably integral with the support, and are prepared by etching a single crystalline wafer material such as silicon. The microprobes are preferably pyramidally shaped. The target cells can be contacted with the microprobes <i>in vitro</i> or <i>in situ</i>. The method is applicable to virtually all cell types, and any biological material capable of being introduced into cells described herein.</p>		

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DESCRIPTIONDIRECT INTRODUCTION OF FOREIGN MATERIALS INTO CELLS  
TECHNICAL FIELD

5 The present invention relates to methods for the introduction of biological materials into cells.

BACKGROUND ART

10 The rapid advancement of recombinant DNA technology has created a wide-ranging need for biological scientists to transfer biological substances from one cell to another, and to transfer synthetic biological material into living cells to exert their activity therein. Such materials include proteins, e.g., antibodies or enzymes, pharmaceutically active agents, and more commonly, nucleic acids such as RNA or  
15 DNA. Thus, a variety of chemical and mechanical cell transformation methods have emerged. Some of the more widely practiced methods include direct micro-injection, electroporation, liposome-mediated transformation, cell fusion, cell implantation, biolistics, and viral- and bacterial-mediated transformation.  
20

In the case of biolistic techniques, metal microprojectiles are coated with the foreign substance, and disposed on a microprojectile which is accelerated  
25 toward the cell target. These techniques suffer from several deficiencies. For example, most of the existing biolistic apparatus is bulky, generally immobile and cannot be hand held such as is needed for some veterinary or medical applications. In addition, they are not flexible as to mode of use; rather, they tend to be optimal for only a single mode of use or for a single application. Further, they do not provide the degree of repeatable results that is desired. Biolistic devices have also been criticized in terms of  
30 inadequate velocity control, and excessive gas blast, acoustic shock, velocity debris, and heat and radiant energy. Moreover, extensive cell damage can occur due to excessively high velocity of the microprojectiles  
35

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when they strike the cells and/or by high-pressure gas impinging directly on the cells. Most biolistic apparatus is limited to one-shot at the target. That is, it is not possible to fire multiple shots of the foreign biological substance at the same target or at different targets in rapid succession.

Cell implantation techniques generally involve creating a small hole in a single living cell with the aid of a fine needle under an optical microscope, thereby allowing DNA fragments to enter the cells through the hole. This method, however, requires skilled manipulation of the needle, and is quite tedious and laborious. Another implantation method involves the steps of precipitating DNA in a culture medium, and making use of the phagocytotic properties of living cells to incorporate the DNA. While this method is capable of handling a great number of cells simultaneously, the success rate is quite low.

Virus-induced and chemical-induced fusion methods also have many shortcomings, including fusion yield and severe side effects on the fused cells. Further, not all cell types can be fused with the same ease.

The ability to transfer exogenous genetic material into higher plants promises to provide enhanced opportunities for agricultural scientists to increase food production. Microinjection of DNA has been practiced in both animal and plant cells. This technique, however, can be applied to only one cell at a time. Other plant transformation techniques have taken advantage of the plant pathogen Agrobacterium tumefaciens, which has the ability to transfer a portion of the DNA from an endogenous Ti (tumor-inducing) plasmid into an infected plant cell. Agrobacterium-mediated plant cell transformation has worked reasonably well in many model crop species, such as tobacco, petunia and carrot. Nonetheless, it has significant limitations. The first is that the

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mediation can be only done on an individual cellular level, typically with somatic tissues, which then must be regenerated artificially into a whole plant. Second, the natural host range of Agrobacterium includes only dicotyledonous plants, and a limited number of monocot species.

In sum, most available cell transformation techniques share common disadvantages. Most of the techniques are painstakingly slow; they use methods which transport materials into, at most, only a few cells at a time; and they lack precise controllability. Most techniques are not universally applicable to a large number of different organisms in different biological classes or kingdoms. For example, chemical methods are generally applicable to procaryotic systems, whereas in eucaryotic systems, both chemical and mechanical methods are used. Existing methods are further dependent upon both the gene which is to be transferred as well as the type of recipient cell. Hence, a strong need remains for a method of transferring foreign biological materials into cells which is fast, uncomplicated, efficient, and which can be routinely and universally applied to large numbers of cells simultaneously.

#### SUMMARY OF THE INVENTION

The present invention provides a method of introducing a biological material of interest into a predetermined target cell population. The method comprises the steps of providing (a) a plurality of microprobes positioned on a support, (b) a solid or quasi-solid mass of the target cells defining an interface with the microprobes, and (c) a biological material at the interface, and then physically contacting the target cells with the microprobes to cause the microprobes to non-lethally pierce the cell membranes of the cells. The biological material is introduced into the cytoplasm of the cells directly via the microprobes, or by passage through the openings or

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perforations in the cell walls and/or membranes created by the microprobes.

5 In a preferred embodiment, a plurality of biologically inert microprobes are integral with the support, and are prepared by etching a wafer, preferably silicon. The wafer is etched in such a manner so as to produce truncated, pyramidal microprobes having a height from about 10 microns to about 300 microns, preferably a height of from about 20  
10 to about 90 microns, a tip width of from about 0.05 to about 10 microns, a base width of from about 30 to about 80 microns, and a distance between any two adjacent microprobes of from about 1.0 to about 20 times the height of the microprobes. The microprobes  
15 can be applied to the target cells *in vitro* or *in situ*.

Another embodiment of the present invention provides a method of introducing a biological material of interest into a predetermined target cell population, comprising the steps of providing in a  
20 liquid medium a target cell population, the biological material, and a plurality of microprobes positioned on a support, the microprobes and support being integral with one another and having been prepared by etching a single crystalline wafer material, and subjecting the  
25 liquid medium containing the target cells, the biological material, and the microprobes to physical motion under conditions sufficient to cause the microprobes to non-lethally pierce the cell walls and/or membranes of the cells, whereby the biological  
30 material enters the cytosol of the cells.

The present invention is applicable to virtually all cell types, and can be practiced with any biological material capable of being introduced into cells in the manner described herein.

35 BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings, the preferred embodiments of the invention are illustrated in which:

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FIG. 1A is a perspective view of a plurality of pyramidal microprobes positioned on a support;

FIG. 1B is an electron photomicrograph of a single pyramidally-shaped microprobe;

5 FIGS. 2A and 2B are cross-sectional views of a preferred system used for carrying out of the present invention;

10 FIG. 3 is a perspective view of another preferred system for carrying out the present invention; and

FIG. 4 is a perspective view of a plurality of barbed-shaped microprobes positioned on a support.

#### BEST MODE OF CARRYING OUT INVENTION

15 Referring now to the drawings, and more specifically to FIG. 1A, there is shown a structure 10 containing a plurality of pyramidal microprobes 12 positioned or disposed on a silicon wafer support or substrate 14. It is preferred that the microprobes are biocompatible. The microprobes and the substrate  
20 preferably constitute an integral, i.e., one-piece, structure.

In a preferred embodiment, the illustrated structure is prepared by one of many variations of bulk micro-machining. A standard silicon wafer is cut so  
25 the plane specified by the Miller indices (100) is coincident with the top surface. The wafer is thoroughly cleaned according to standard procedures, such as a RCA clean. Next, a protective layer is grown or deposited on the top surface of the wafer. For  
30 example, a one-micron thick silicon dioxide layer could be grown on the wafer by wet oxidation at approximately 1000° C for six to twelve hours. In the alternative, a thinner layer of oxide could be grown, and silicon nitride deposited on top of the oxide. Photoresist is  
35 then placed on t p of the wafer. For example, the wafer can be spun, and several drops of photoresist placed n the surface. By correctly controlling the spinning of the wafer, a thin (e.g., usually a micron

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or less) layer of photoresist is left on the top surface of the wafer. The photoresist is then soft baked, at approximately 100° C for ten to thirty minutes, to strengthen the photoresist for handling and subsequent steps.

The photoresist is then patterned. An optical mask having the correct features is used to expose the photoresist. For example, the mask can be brought in contact with the top surface of the photoresist, and ultraviolet light shown through the mask onto the photoresist. The photoresist is then developed, using procedures specified by the particular manufacture of the photoresist. Then the photoresist is hardbaked for approximately one-half hour at approximately 120° C. At this point, photoresist only covers those portions of the wafer that were not exposed to ultraviolet light (or in some cases, only those regions that were exposed to ultraviolet light).

The wafer is then placed in buffered hydrofluoric acid for several minutes to remove the oxide layer present on the wafer, except where the wafer is protected by the remaining photoresist. If the wafer is also protected by silicon nitride, a method of removing the silicon nitride, such as reactive ion etching, needs to be used.

The remaining photoresist is removed off the top surface of the wafer, leaving the silicon oxide and/or silicon nitride ( $\text{SiO}_2$  /  $\text{SiN}$ ) protecting selected regions of the wafer. For the micro-point fabrication, these protected regions of  $\text{SiO}_2$  /  $\text{SiN}$  are squares with their sides orientated along the [110] directions. Alternatively, the protected regions could be squares orientated along the [100] directions with corner compensation arms of  $\text{SiO}_2$  /  $\text{SiN}$  extending away from the corners of the squares. This corner compensation allows taller micro-probes to be fabricated.

The wafer is placed in an anisotropic etching solution. Isotropic etching could also be used. A



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solution of approximately one part KOH and three parts of distilled water is preferred. The wafer is etched until the  $\text{SiO}_2$  /  $\text{SiN}$  protective regions start to float off the wafer into the water. This indicates the protective coating has been completely under etched. The wafer is now cleaned, and cut into appropriately shaped chips.

In the a more preferred embodiment, the illustrated structure is prepared by the anisotropic etching of {100} silicon wafers substantially as disclosed in Offereins et al., Sensors and Actuators A, 25-27:9-13 (1991), with modifications.

The result of cutting (etching) the top surface of the silicon wafer in the {100} direction is the formation of discrete, four-sided truncated pyramids formed by {411} planes, i.e., the microprobes. Upon completion of the etching process, the substrate 14 preferably has a final thickness of from about 200 to about 550 microns.

Microprobes 12 have a half angle  $h$  of about  $13^\circ$  (not illustrated). That is, each face is disposed at an angle of about  $13^\circ$  to the central axis of the pyramid. Stated differently, the included angle between any two oppositely directed faces of the pyramidal structure is about  $25^\circ$ . In general, the dimensions of the microprobes and the distance between any two adjacent microprobes on support 14 are selected so as to maximize the number of cells in the target cell population that are pierced by the microprobes in a single application. As illustrated in FIG 1B, the height  $h$  of the microprobes will usually range from about 10 microns to about 300 microns. The preferred height is from about 20 to about 90 microns, more preferably about 80 microns. The tip width  $t$  of the microprobes will usually vary from about 0.05  $t$  about 10 microns, and is preferably no more than about 1 micron. The base width,  $b$ , i.e., the linear dimension along an edge of the microprobes at the substrate or

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support 14, is from about 30 to about 80 microns, and the distance between microprobes is from about 1.0 to about 20.0 times the height of the microprobes, e.g., from about 10 to about 3000 microns, and preferably from about 80 to about 800 microns. Thus, a 5 millimeter square silicon wafer typically will contain about 500 microprobes. These dimensions can be varied by adjusting the process parameters, e.g., dimensions of mask, etching time, etchant type concentration of etchant and etching time, accordingly. See Petersen, "Silicon as a Mechanical Material", Proc. IEEE 70(5):420-457 (1982).

Turning now to FIG. 2A, system 20 features opposing plates 22 and 22', connected by flexible linkages 25 and 25'. Plate 22' contains a recess 23 bounded by a wall having an edge 29. This recess is adapted to receive structure 10 and a solution of biological material 24. Thus, when structure 10 is disposed in recess 23, substrate 14 is recessed from edge 29. However, the tips of microprobes 12 protrude beyond edge 29. The surface of plate 22 facing the microprobes is attached to flexible material 26. The flexible material is easily deformed (deformable), highly elastic, and resilient so as to conform to the surface of the solid or quasi-solid mass of the target cells. Examples include silicon rubbers and gels, and foam rubbers. The biological material, in this case a tobacco plant leaf 28, is disposed atop the microprobes, thus defining an interface between the microprobes and the tobacco cells 28'. A solution of biological material 24 is present at this interface. Alternatively, the leaf may be attached to flexible material 26.

As illustrated in FIG. 2B, upon the application of sufficient force urging plates 22 and 22' towards one another, the microprobes are caused to pierce individual cells 28' of leaf 28, rendering the cells temporarily permeable such that biological

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material solution 24 is caused to enter (e.g., diffuse into) the cytosol of the cells. Of course, to the extent that biological material is retained on the microprobes (as described in greater detail below), the material is delivered into the cytosol by the microprobes per se. By the term "sufficient" force, it is meant that the flexible material 26 presses the biological material until the tips of the microprobes penetrate the cells and force leaf 28 approaching the surface of substrate 14. This process can be repeated several times, each time changing the position the leaf 28 relative to the probes 12 so that different injection locations are produced, thus maximizing the number of cells pierced.

In FIG. 3, structure 30 contains a handle 31 attached at an appropriate angle to support 33. The handle and support can also be integral with one another. That is, an appropriately shaped structure can be made from one piece. Substrate 34 having pyramidal microprobes 32 disposed thereon is attached to the bottom surface of support 33. This embodiment of the present invention is advantageously used to apply the microprobes directly to the target cells *in situ*, e.g., *in vivo*. Examples of target cells particularly well suited to the application of the microprobes in this fashion include animal skin (epidermal cells), animal and human internal tissues, and plant tissue. In the case of internal tissues, structure 30 can be used during the course of a surgical or endoscopic procedure as an adjunct in gene therapy. In this embodiment, the biological material may be applied directly to the target cell population prior to the application of the microprobes, or onto the microprobes themselves as described herein. Those skilled in the art will appreciate that size and shape of the handle and support can be varied to accommodate the particular use.

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FIG. 4 illustrates another preferred embodiment of the present invention, wherein a plurality of barbed microprobes 42 are positioned on silicon substrate 44. Each barb 42 is comprised of a support 42', and a head 42'' which is disposed upon the support 42'. The barbed microprobes are prepared by the isotropic etching of [100] oriented silicon wafers as described in Han et al., "Mating and Piercing Micro-mechanical Structures for Surface Bonding Applications," in The Proceedings of the IEEE Micro Electro Mechanical Systems Workshop, January, 1991, Nara Japan, pp. 253-258. Briefly, the silicon wafer is cleaned and oxidized, and then patterned into an array of small squares. The wafer is then etched in KOH and isopropyl alcohol. The oxide mask is then stripped and a second silicon dioxide film is grown. This film is patterned into an array of Greek crosses, and a second KOH etch is applied to remove a portion of the underlying silicon. An isotropic etch is then used to remove additional material from under the thus-formed microbarbs. The barbed microprobes 42 have a height equal to about twice that of the microprobes 12 illustrated in FIG. 1. The dimensions i.e., tip width, base width and height, of each of the heads 42'' and support 42', are approximately equal to those of a single pyramidal microprobe described above. When the method of the present invention is practiced using barbed microprobes, cell death is minimized by providing a lateral force to substrate 44 while the heads of the microprobes are in contact with the cytosol of the target cells, so as to break off heads 42'' from supports 42'. In this manner, the step of removing the barbed microprobes from the cells and the concomitant risk of causing cell death is minimized or obviated. The lateral force is typically in the range of from about 0.001 to about 1.0 lb/microprobe.

There are numerous permutations of the preferred embodiments. The microprobes can be prepared

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using any substance which can be formed into a desired shape and which is capable of piercing cell walls and cell membranes, and preferably which will maintain its structural integrity over the course of several applications to the target cells. Biocompatible materials are preferred for these purposes, in addition to their non-reactivity with and non-toxicity to cells. In regard to etchable materials, the microprobes and substrates are prepared from any single crystalline material that can be isotropically etched or anisotropically etched relative to crystallographic planes. Examples include quartz and gallium. The etching process can also be varied to produce needle-like or whisker-like microprobes. The Petersen et al., *supra*. Those skilled in the art will appreciate, however, that the support-based microprobes can be prepared or replicated in accordance with art-recognized techniques other than etching such as molding or metal plating, using non-etchable materials such as ceramics, plated metals and plastics. See, e.g., Erfeld et al., Fabrication of Microstructures Using the LIGA Process, Kernforschungszentrum Karlsruhe GmbH, Karlsruhe, Federal Republic of Germany.

The biological materials can be applied to the microprobes and/or support or substrate in several ways. For example, the substrate or support can be adapted, e.g., recessed, to hold a liquid solution of the biological material. A film or coating of the biological material may also be deposited or otherwise applied directly onto the microprobes in accordance with standard techniques. Such a composition may be prepackaged. Moreover, surface tension alone typically will hold the biological material in place between the probes. Although the invention is not limited to any particular theory of operation, it is believed that because the microprobes are closely spaced, any liquid containing the material which wets the substrate surface will be trapped as a meniscus between the

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microprobes. This effect can be enhanced by adding a wetting agent to the solution of biological material, or to the medium in which the method is carried out. In the case of silicon, again while not being bound to any particular theory of operation, it is believed that when the silicon surfaces of the microprobes and substrates are exposed to air, an oxide is formed which is hydrophilic in nature and thus causes any aqueous biological material solution to more strongly adhere thereto. Further, the microprobes and/or substrate can be physically pretreated, e.g., roughened or chemically pretreated, e.g., with a porous material to enhance the "adhesion" between the biological material solution and the probes and substrate.

The conditions in which the physical contact between the microprobes and target cells occurs can be varied widely. For example, the solid or quasi-solid target cell population may be provided in a liquid, i.e., hydroponic, or a misting, i.e., aeroponic environment. In addition, the injection location and the penetration depth of the microprobes can be accurately controlled on both two dimensional and three dimensional planes. Two dimensional controllability is achieved simply by conducting multiple applications of the probes to the cells, each time positioning the target cell population relative to the microprobes such that different injection locations are produced. Three dimensional controllability can be used to deliver biological material to exact locations in the cells. The control of the biological material delivery can be achieved based on force or displacement feedback. In the case of force feedback, the force required to penetrate a target cell membrane or cell wall to a specific depth is determined and this force is then applied to the microprobe-mediated injection system such as the transfusion clamp illustrated in Figs. 2A and 2B. On the other hand, if the thickness or cell membrane of cell wall of a target cell is known, then

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the displacement of the microprobes can be manipulated to stop immediately or at any desirable depth after the microprobe tips are exposed to cytosol of the target cells. To improve the biological material delivery efficiency of the microprobes, the penetration depth of the microprobes can be controlled based on the knowledge of cell size and/or structure or strength of target cell membrane or cell wall. Although the invention is not limited to any particular theory of operation, it is believed that in the case where contoured, e.g., pyramidal or barbed, microprobes are used, displacement control is facilitated because force increases with the displacement of the probes in the cells.

To further increase the uptake of the biological material by the target cells, electric pulses of a voltage sufficient to enhance the reversible permeabilization of the cells can be applied to the microprobes (which may also include the substrate) and/or the medium. Typical voltages are in the range of about 3 kilovolts to about 20 kilovolts. See, e.g., U.S. Patent No. 4,849,355. The method can also be conducted in an osmotic gradient. Thus, the present invention may be practiced in conjunction with other techniques for introducing biological materials into cells, or with techniques which facilitate the introduction of the material once the cells are permeabilized by the microprobes. Thus, the present invention may be used in conjunction with other techniques for introducing biological materials into cells, or techniques which facilitate the introduction of the material once the cells were permeabilized by the microprobes.

Any biological material, which when delivered to the cyt sol of a cell is capable of exerting its intended effect, can be used in the present method. Suitable biological materials include non-proteinaceous substances such as organic and inorganic

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chemicals, e.g., insecticides, diagnostic chemicals, e.g., detectable labels such as radio- or fluorescent-labeled molecules and pharmaceutically active substances, e.g., drug such as anti-inflammatories and antibiotics; proteins such as antibodies, hormones, growth factors and enzymes; and nucleic acids such as DNAs, particularly DNAs which encode a protein of interest. Nucleic acids are preferred.

The present method may also be practiced with virtually any cell type, including both procaryotic, e.g. bacterial, and eucaryotic cells, e.g., animal, human, plants and yeast. Examples of eucaryotic cells include animal and plant cells, the latter including both monocotyledonous and dicotyledonous cells, and protoplasts thereof. Preferred plant species include cereal crop species such as rice, corn, wheat, sorghum, barley, soybean, potato, tomato and legumes. In general, the method is applicable to any cell type whose cell membranes (and in the case of plants, cell walls) can be pierced by the instant microprobes. The method of the present invention has been illustrated using target cells *in vitro*. However, the microprobes can also be applied to the cells *in situ*, as described above. In addition, the definition of "solid or quasi-solid target cells" for purposes of the present invention is meant to include multi-cellular living organisms and tissues of living organisms.

In yet a further embodiment of the present invention, a method of introducing a biological material of interest into the cytosol of a target cell is provided wherein a solid or quasi-solid mass of cells is not required. In this embodiment, the target cells, biological material and the support-based microprobes are provided in a liquid medium, with the support being anchored or secured, e.g. to an interior surface of a container holding the liquid mixture. The support-based microprobes are prepared by etching a wafer, preferably silicon, as described above. The



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liquid medium containing the target cells, the biological material, and the microprobes is then subjected to physical motion for a period of time and under conditions sufficient to cause the microprobes to non-lethally pierce the cell walls and/or cell membranes of the cells, whereby the biological material is caused to enter the cytosol of the cells. The liquid medium may be agitated by stirring, vortexing, or by an appropriate mechanical, magnetic or electrical force as described, for example, in U.S. Patent 5,302,523 to Coffee et al.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Unless otherwise indicated, all percentages are by weight.

#### EXAMPLE 1

##### Fabrication Process for Microprobes

Polished {100} oriented silicon wafers were cleaned in a mixture of hydrochloric acid (HCl) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to remove ionic contaminants. The wafers were dipped in a 10:1 hydrofluoric acid:water solution to remove the native oxide layer, then treated with sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and H<sub>2</sub>O<sub>2</sub> to grow a thin chemical oxide film. The samples were oxidized for 8 hours in an electrically-heated quartz furnace tube maintained at 1000°C in pure oxygen bubbled through deionized water resulting in an SiO<sub>2</sub> film approximately 1.0 μm thick. A single-mask lithographic sequence was employed to pattern the wafers. Hexamethyl disilazane was applied to the obverse face of the samples followed by AZ4210 photoresist spun at 6000rpm for 60 seconds. The photoresist solvent was expelled with a 20 minute bake at 90°C. An emulsion mask containing 10 μm<sup>2</sup> patterns in a clear field was used in a contact aligner to expose the photoresist. The edges of the patterns aligned to the {011}

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directions. After exposure, the samples were developed in a 3:1 solution of deionized water and AZ400K for 60 seconds, then hardbaked for 30 minutes at 120°C. The developed photoresist pattern was then transferred to the SiO<sub>2</sub> film by etching in buffered hydrofluoric acid. Etch completion was determined by observation of the hydrophobic surface transformation on the wafer backsides. The wafers were subjected to cascade rinsing in deionized water followed by photoresist removal in successive baths of acetone, methanol, and deionized water.

The microbarbs are formed by anisotropic silicon etching in an aqueous solution of KOH at 85°C. The etchant attacks the exposed {100} silicon surface, but etches the protective SiO<sub>2</sub> pattern very slowly. These etching conditions result in undercutting of the convex corners of the mask, exposing {411} oriented planes. These facets etch rapidly until they meet the center resulting in a sharp point and mask liftoff. The etch timing is monitored to ensure that the microbarbs are not further eroded.

#### EXAMPLE 2

##### Transient Expression of DNA in *Nicotiana Tabacum* After Microprobe-Mediated Transformation

To demonstrate that a biological material can be deposited into cells by this method, an experiment was carried out using Tobacco (*Nicotiana Tabacum* W3) leaf tissues. The DNA of interest was the plasmid pB 121 which contains a kanamycin resistance gene and a GUS assay sensitive protein (glucuronidase). The microprobes were prepared according to Example 1. The silicon substrate had a dimension of 5 mm by 8 mm and contained approximately 200 25-μm height microprobes. The probes were soaked in 95% ethanol for 20-30 minutes and washed 3 times with autoclaved distilled deionized H<sub>2</sub>O, then air dried thoroughly. A DNA mix was made using 5 μg DNA in TE buffer, 12.5 μl of 2.5 M CaCl<sub>2</sub>, and 5 μl of 0.1 M spermidine free base. The epidermal

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layer of the severed tobacco leaf was aseptically removed using forceps. To prepare the microprobes for the plant material, 2.5  $\mu$ g of DNA preparation were placed on the microprobes and the plant material was placed on the microprobes. A cotton swab was used to rub the back-side of the leaf sample to encourage the penetration by the microprobes. The plant material was then removed from the microprobes and then cultured for 72 hours on a regenerative tobacco medium.

To evaluate the effectiveness of the technique, one experimental group and three control groups were conducted. The experimental group was applied to the DNA coated microprobes. The first control group was applied to microprobes that were without DNA solution. The second control group was made to contact the DNA solution, but without the microprobe treatment. The third control group was neither exposed to DNA solution nor was treated with the microprobes. Tobacco tissues were examined by applying a GUS assay to evaluate the amount of DNA taken up by the tobacco cells, following the procedures described in Jefferson, GUS Gene Fusion System User's Manual, Cambridge (July 1987). Successful plasmid uptake by the cells results in the presence of typical blue staining. The experimental group showed positive transient symptoms (i.e., dark blue-green dots), while an absence of such staining was observed in all three control groups.

### EXAMPLE 3

#### Nematode Transformation

The microprobe-mediated transformation was used to generate transgenic nematodes. pPCZ1 and pRF4 were the DNAs used. pPCZ1 contained the C. elegans 16kDa heat shock promoter fused to the E. coli B-galactosidase gene. pRF4 contained a 4Kb EcoR1 genomic fragment of C. elegans encoding the rol-6 (Su1006) collagen gene as described in Kramer et al., Mol. Cell. Biol. 10:2081-89 (1990). The rol-6 gene was provided

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by Peter Candide, University of British Columbia, Vancouver, B.C.

Heterorhabditis bacteriophora HP88 were collected at 4-6 eggs stage from lipid agar seeded with photorhabdus luminescence in double distilled sterile water and washed three times with sterile water. The microprobes of Example 1 were placed with the microprobes pointing up on the surface of a lipid agar plate seeded with photorhabdus luminescence. A DNA mix containing 5  $\mu$ g DNA in TE buffer, 12.5  $\mu$ l of 2.5 M  $\text{CaCl}_2$ , and 5  $\mu$ l; of 0.1 M spermidine-free base. Ten  $\mu$ l of DNA mix were pipetted onto the microprobes to coat the tips with DNA. After 3 minutes, 10  $\mu$ l of a highly concentrated nematode suspension (approximately 200-250 nematodes) were pipetted on top of the microprobes and left at room temperature for 8 to 10 minutes. Most nematodes crawled off the microprobe array onto the media. The array was then removed from the plate, and the agar plate containing nematodes was incubated at 25°C, until the injected nematodes produced progeny. Control experiments, wherein a microprobe array without DNA was used, were also conducted.

After the incubation period, hermaphrodite progeny were examined for roller phenotypic expression of the transformants. Progeny were assayed for expression of the hsp-16-lacZ fusion gene by heat shocking for 2 hours at 33°C as described in Stringham et al., Mol. Biol. Cell. 3:221-33 (1992). The nematodes were then permeabilized by lyophilization and acetone treatment and incubated at 25°C in a standard histochemical containing 3% X-gal as described in Fire EMBO J. 5:2673-80 (1986).

Nematodes were scored for the presence of typical blue staining of b-galactosidase; 8% of the total progeny showed b-galactosidase expression in the first (F1) generation. No staining was observed in the control groups. Although the nematodes were heritable transformed individuals exhibited a non-Mendelian

pattern of inheritance similar to those of C. elegans (extrachromosomal transformants), there was no genome integration.

#### INDUSTRIAL APPLICABILITY

5           The present invention provides a simple, economical and precise method for introducing a biological material of interest into a predetermined target cell population, *in vitro* or *in situ*. Thus, it is useful in both animal and plant applications, including therapeutics and plant biotechnology. The present invention can accommodate virtually all cell types and biological materials, and offers several additional advantages over known biological material, e.g., gene, transfer methodologies. First, the location of the introduction of the foreign biological materials and the penetration depth can be precisely controlled by varying the dimensions of the microprobes, the injection pattern or both, thus "customizing" the method to any given target cell population. Second, cell damage is minimized; the degree of penetration of each probe into the cell, and the size of the holes or perforations in the cell membrane, are controlled by the geometry of the microprobes. Third, the method is simple; it does not require complicated preparatory steps associated with biolistic techniques. Fourth, the method is economical--the microprobe-substrate structure is inexpensive to manufacture, and can be easily sterilized and/or disposed of. Fifth, a larger number of cells are treated simultaneously, compared with prior art microinjection techniques which are limited to the treatment of single cells at a time.

35           All publications cited in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein incorporated by reference to the same extent as if each individual publication were

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specifically and individually indicated to be incorporated by reference.

Further modifications of the invention described herein become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

**CLAIMS:**

1. A method of introducing a biological material of interest into a predetermined target cell population, characterized by the steps of:

5 providing (a) a plurality of microprobes positioned on a support, (b) a solid or quasi-solid mass of the target cells defining an interface with the microprobes, and (c) the biological material at the interface; and

10 physically contacting the cells with the microprobes to cause the microprobes to non-lethally pierce the cell membranes of the cells.

2. A method according to claim 1, wherein the microprobes are pyramidally shaped.

15 3. A method according to claim 1, wherein the microprobes are needle-shaped.

4. A method according to claim 1, wherein the microprobes are barbed shaped.

20 5. A method according to any of claims 1-4, wherein the microprobes and the support are integral with one another, and are prepared by etching a wafer.

6. A method according to claim 5, wherein the wafer comprises a single crystalline material.

25 7. A method according to claim 5, wherein the etching is anisotropic.

8. A method according to claim 5, wherein the etching is isotropic.

9. A method according to claim 5, wherein the material is silicon.

30 10. A method according to claim 5, wherein the material has a top surface cut in the {1 0 0} direction.

35 11. A method according to claim 5, wherein the microprobes are formed by etching to the {411} planes of the wafer.

12. A method according to any of claims 1-4, wherein the microprobes have a height of from about 10 microns to about 300 microns.

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13. A method according to any of claims 1-4, wherein the microprobes have a height of from about 20 to 90 microns.

14. A method according to any of claims 1-4, wherein the microprobes have a tip size of from about 0.5 to about 10 microns.

15. A method according to any of claims 1-4, wherein the microprobes have a base size of from about 30 to about 80 microns.

16. A method according to any of claims 1-4, wherein the distance between the microprobes is from about 1.0 to about 20 times the height of the microprobes.

17. A method according to any of claims 1-4, wherein the support has a thickness of from about 200 to about 550 microns.

18. A method according to claim 1, wherein the biological material is an organic or inorganic chemical.

19. A method according to claim 18, wherein the biological material is a pharmaceutical agent.

20. A method according to claim 1, wherein the biological material is a protein.

21. A method according to claim 20, wherein the protein is an enzyme.

22. A method according to claim 1, wherein the biological material is a nucleic acid.

23. A method according to claim 22, wherein the nucleic acid contains a DNA molecule encoding a protein of interest.

24. A method according to claim 1, wherein the predetermined target cell population contains eucaryotic cells.

25. A method according to claim 24, wherein the eucaryotic cells are animal cells.

26. A method according to claim 25, wherein the animal cells are human cells.



27. A method according to claim 25, wherein the animal cells are epidermal cells.

28. A method according to claim 24, wherein the eucaryotic cells are plant cells.

5 29. A method according to claim 28, wherein the plant cells are monocotyledonous cells.

30. A method according to claim 28, wherein the plant cells are dicotyledonous cells.

10 31. A method according to claim 1, wherein the predetermined target cell population contains procaryotic cells.

32. A method according to claim 1, wherein said step of physically contacting characterized by the step of contacting the microprobes with the target  
15 cells *in situ*.

33. A method according to claim 1, wherein said step of contacting is repeated at least twice, wherein prior to each repetition the disposition of the target cells relative to the microprobes is changed  
20 such that upon said contacting step, a different injection pattern over the previous repetition is achieved.

34. A method according to claim 1, wherein said step of providing the biological material is  
25 characterized by applying the biological material to the microprobes, the support or both the microprobes and the support.

35. A method according to claim 1, wherein said step of providing the biological material is  
30 characterized by applying the biological material to the target cells.

36. A method according to claim 1, wherein said step of physically contacting is characterized by applying a predetermined force to the support.

35 37. A method according to claim 1, wherein said step of providing is characterized by providing the plurality of microprobes positioned on a first

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support and the target cells positioned on a second support.

38. A method according to claim 37, wherein said step of physically contacting is characterized by applying a predetermined force to the first support, the second support, or to both the first and second supports.

39. A method according to claim 1, further characterized by the step of applying electric pulses to the microprobes.

40. A method of introducing a biological material of interest into a predetermined target cell population, characterized by the steps of:

providing in a liquid medium the target cells, the biological material, and a plurality of microprobes positioned on a support, the microprobes and support being integral with one another and having been prepared by etching a single crystalline wafer material, and

subjecting the liquid medium containing the target cells, the biological material, and the microprobes to physical motion under conditions sufficient to cause the microprobes to non-lethally pierce the cell membranes of the cells.

41. A method according to claim 40, further characterized by the step of applying electric pulses to the medium.

42. A composition of matter, characterized by:

a plurality of microprobes positioned on the surface of a support, and a biological material disposed on the microprobes, the surface of the support, or both the microprobes and the surface of the support.

43. A composition of matter, according to claim 42, wherein said support and said microprobes contain silic n.

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44. A composition of matter, according to claim 42 or claim 43, wherein said biological material contains DNA.

5 45. A nematode transformed with a recombinant DNA molecule, said molecule characterized by a promoter which in its native state is associated with a C. elegans heat shock protein gene, operably linked to an E. coli  $\beta$ -galactosidase structural gene.

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FIG. 1A

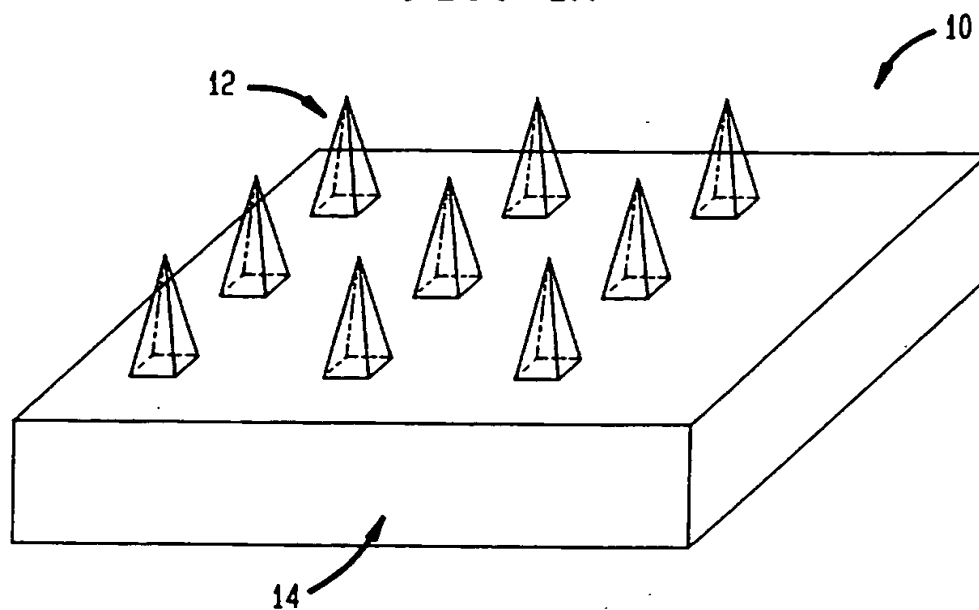
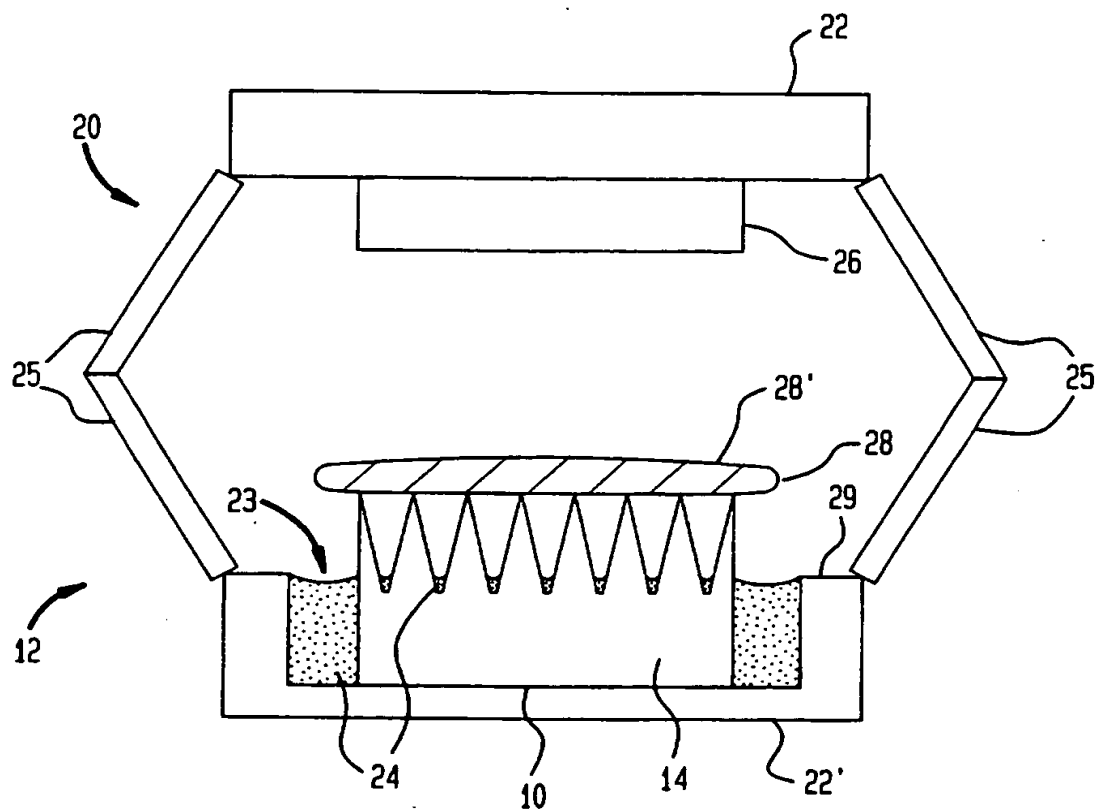
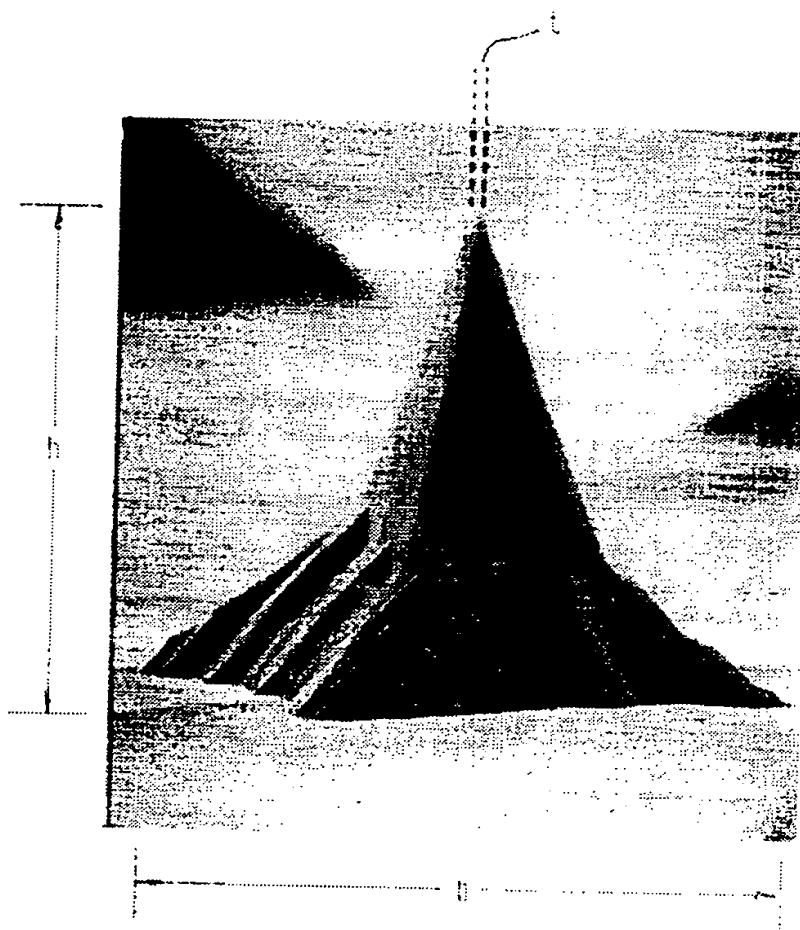


FIG. 2A



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FIG. 1B



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FIG. 2B

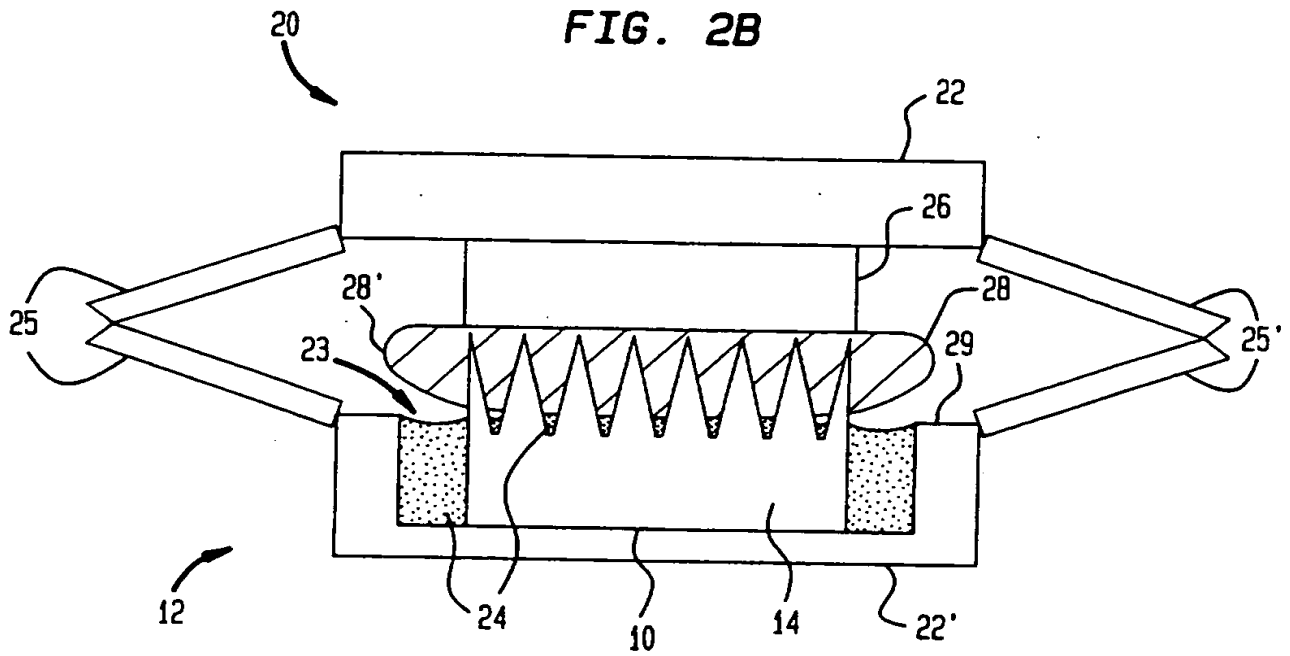


FIG. 3

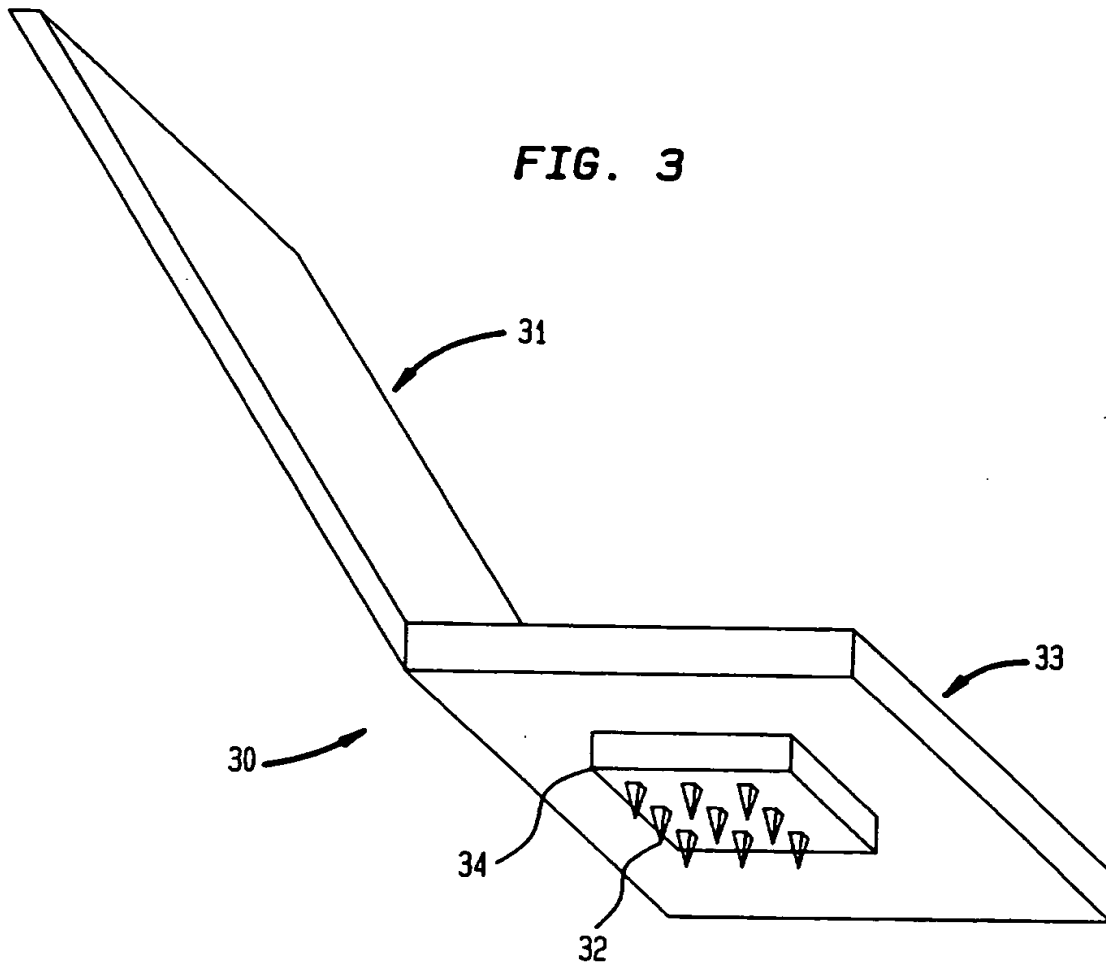
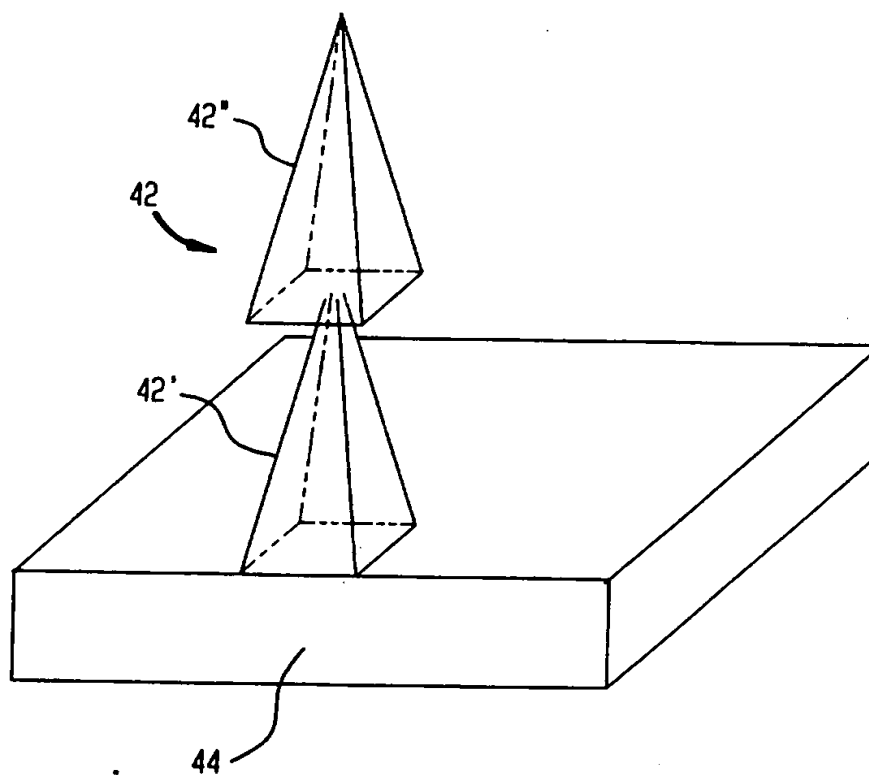


FIG. 4



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/12381

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 15/06, 13/00; C12M 1/04, 3/04; C12N 15/85, 15/82; A01K 29/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/172.1, 172.3, 173.1, 173.6, 285.1, 313, 320.1, 240.1, 240.2, 240.4; 119/6.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Molecular Biology of the Cell. Volume 3, issued February 1992, Stringham et al., "Temporal And Spatial Expression Patterns of the Small Heat Shock (hsp16) Genes in Transgenic Caenorhabditis elegans", pages 221-233, entire document.	45
Y	US, A, 5,302,523 (COFFEE ET AL.) 12 April 1994, see entire document, especially columns 1-4 and the claims.	1-45
Y	Sensors And Actuators, Volume 25-27, issued 1991, Offereins et al., "Methods for the Fabrication of Convex Corners in Anisotropic Etching of (100) Silicon in Aqueous KOH", pages 9-13, see entire document.	1-45

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be part of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* &	document member of the same patent family

Date of the actual completion of the international search

15 DECEMBER 1995

Date of mailing of the international search report

26 JAN 1996

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Form PCT/ISA/210 (second sheet)(July 1992)\*



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/12381

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E, X ----- Y	US, A, 5,457,041 (GINAVEN ET AL.) 10 October 1995, see the entire document.	1 ----- 2-45
Y	US, A, 5,262,128 (LEIGHTON ET AL.) 16 November 1993, see the entire document, especially the figures.	1-45
Y	Proceedings IEEE Micro Electro Mechanical Systems. Conference title: "An Investigation Of Micro Structures, Sensors, Actuators, Machines And Robots", issued 1991 (conference held 30 January to 02 February 1991), Han et al., "Mating And Piercing Micromechanical Structures For Surface Bonding Applications", pages 253-258, see entire document.	1-45
Y	US, A, 4,849,355 (WONG) 18 July 1989, see entire document.	36, 38, 39, and 41

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/12381

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

800/2; 435/172.1, 172.3, 173.1, 173.6, 285.1, 313, 320.1, 240.1, 240.2, 240.4; 119/6.7

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System - USPAT and JPOABS

Dialog - BIOSIS, PASCAL

Search terms: micromechanical, silic?, transform?, transfect?, transduce?, plant?, animal?, microprob?, cell? etch?, conic?, pyramid?, barb?, plate?, transgen?, photoresist?, photoetch?

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-44 drawn to a method of transfection and a composition of matter for transfection.

Group II, claim 45 drawn to a transformed nematode.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the transfection method and the composition of matter used in the transfection method has a special technical feature (the specific method used in transformation) which is not present in the claim drawn to a transformed nematode (that may have been transformed by any method). The transformed nematode has a special technical feature (the exact product limitations such as a specific promoter and gene) which are not present in the process of transformation (and composition of matter) of Group I. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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